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T. Lewis, N.J. Loman, L. Bingle, P. Jumaa, G.M. Weinstock, D. Mortiboy, M.J. Pallen,

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Abstract: Shared care of military and civilian patients has resulted in transmission of multidrug-resistant *Acinetobacter baumannii* (MDR-Aci) from military casualties to civilians. Current typing technologies have been useful in revealing relationships between isolates of *A. baumannii* but they are unable to resolve differences between closely related isolates from small-scale outbreaks, where chains of transmission are often unclear. In a recent hospital outbreak in Birmingham, six patients were colonised with MDR-Aci isolates indistinguishable using standard techniques. We used whole-genome sequencing to identify single nucleotide polymorphisms in these isolates, allowing us to discriminate between alternative epidemiological hypotheses in this setting.

Keywords: *Acinetobacter baumannii*; Epidemiology; Multidrug resistance; Single nucleotide polymorphism; Whole-genome sequencing; 454 pyrosequencing

Use of high-throughput whole-genome sequencing to dissect the epidemiology of *Acinetobacter baumannii* isolates from a hospital outbreak

*Dr Tom Lewis[a], *Dr Nicholas J. Loman[b], Dr Lewis Bingle[b], Dr Pauline Jumaa[a], Professor George M. Weinstock[c], Dr Deborah Mortiboy[a] and Professor Mark J. Pallen[b,#]

[a]Department of Medical Microbiology, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK

[b]Centre for Systems Biology, University of Birmingham, Birmingham, UK

[c]Department of Genetics, Washington University School of Medicine, St. Louis, Missouri, USA

*These authors contributed equally to this work

Corresponding author. Address: Centre for Systems Biology, University of Birmingham, Birmingham, B15 2TT, UK. Tel: +44 121 414 7163.

E-mail: m.pallen@bham.ac.uk

Running title High-throughput sequencing of *Acinetobacter* isolates

Summary

Shared care of military and civilian patients has resulted in transmission of multi-drug-resistant *Acinetobacter baumannii* (MDR-Aci) from military casualties to civilians. Current typing technologies have proven useful in revealing relationships between *A. baumannii* isolates. However, they are unable to resolve differences between closely related isolates from small-scale outbreaks, where chains of transmission are often unclear. In a recent hospital outbreak in Birmingham, six patients were colonized with MDR-Aci isolates indistinguishable using standard techniques. We have used whole-genome sequencing to identify single nucleotide polymorphisms (SNPs) in these isolates, allowing us to discriminate between alternative epidemiological hypotheses in this setting.

Introduction

In the United Kingdom, military casualties are usually repatriated to Selly Oak Hospital, Birmingham, where they are cared for alongside civilian patients. Military patients from Iraq and Afghanistan are often colonized with strains of multi-drug-resistant *A. baumannii* (MDR-Aci), which can spread to civilian patients and health-care workers.[1-6] Molecular typing systems, such as pulsed-field gel electrophoresis (PFGE) and variable-number tandem repeat (VNTR) analyses, have provided evidence of multiple concurrent or successive clonal outbreaks in hospitals across the UK.[7-8] However, these methods have been unable to provide sufficient resolution to determine chains of transmission within apparently clonal outbreaks. Nor can they provide detailed information on patterns of spread (e.g. the influence of "super-shedders", environmental persistence, staff carriage or infection control practices), even though these questions are important when considering where to focus finite infection control resources.

A recent MDR-Aci outbreak in Selly Oak Hospital illustrates some of these problems. Four military patients, admitted over a five-week period, were each found to be colonised with MDR-Aci a few days after admission. Subsequently, indistinguishable isolates were recovered from two civilian patients on the same unit. Molecular typing distinguished isolates from this outbreak from those recovered from a similar outbreak in 2007 (same PFGE type, different VNTR type). However, such approaches were unable to shed light on transmission events within the 2008 outbreak itself.

Whole-genome sequencing represents the ultimate molecular typing method for bacteria, because it samples the entire collection of genetic information within each isolate. Pioneering work on the "Amerithrax" strain of *Bacillus anthracis* illustrated the utility of this approach[9] as long ago as 2002. However, until recently, the cost and technical complexity of bacterial whole-genome sequencing placed it beyond the reach of the average diagnostic laboratory or academic research group. This has changed in the last couple of years with the advent of "high-throughput sequencing"—an umbrella term for several competing technologies that deliver genome sequences around one hundred times more quickly and more cheaply than conventional sequencing approaches (see recent review by Metzker[10]). All such technologies do away with the need for cloning of DNA in biological systems and instead rely on massively parallel *in vitro* amplification of template molecules attached to a solid surface.

Several recent studies have shown that analysis of single nucleotide polymorphisms (SNPs) in bacterial genomes provides a means of determining relatedness between epidemiologically linked isolates and tracking bacterial evolution over periods of months to years.[11-16] Furthermore, the massive depth of coverage provided by high-throughput sequencing means that, when looking for rare genomic changes, it becomes efficient to pool samples and identity variable loci by polymorphisms within the consensus sequence. SNPs can then be quickly and easily assigned to individual isolates by a small number of confirmatory PCRs.

Despite the promise of these new technologies, at the outset of this study, it remained unclear whether MDR-Aci lineages associated with individual patients harbour SNPs capable of providing useful epidemiological information. We therefore applied one particular high-throughput sequencing technology—454 pyrosequencing—to isolates from our outbreak in the hope of gaining additional epidemiological information.

Methods

Microbiology

A. baumannii isolates were obtained from routine clinical samples. Bacterial identification and antibiotic susceptibility testing was performed on the Vitek 2 system according to the manufacturer's instructions (bioMérieux, Basingstoke, UK), supplemented by CLSI-recommended confirmatory testing. Isolates were frozen on beads and stored at -20°C. Isolates M1, M2, M3, M4 were obtained from wound swabs from military patients. Isolates C1 and C2 were obtained from sputum cultures from civilian patients. Isolates C1-2a and C1-2b were distinct colonies from the same wound specimen, taken two weeks after the initial isolate from civilian patient C1. Isolates C1-3a and C1-3b were isolates from a different wound specimen, taken at the same time as isolates C1-2a and C1-2b. Multidrug resistance was defined as resistance to ≥ 3 classes of antibiotics (quinolones, extended-spectrum cephalosporins, ß-lactam/ß-lactamase inhibitor combinations, aminoglycosides and carbapenems). MDR-Aci isolates were sent to the Laboratory of HealthCare Associated Infection for speciation and PFGE and VNTR analysis. Antibiotic sensitivities were confirmed by agar dilution methods in two isolates (M1 and C1).

Isolation of genomic DNA and 454 sequencing

Genomic DNA was obtained from colony-purified MDR-Aci, using the DNeasy DNA extraction kit (Qiagen, Crawley, UK). DNA was sequenced using 454 Titanium protocols (Roche, Welwyn Garden City, Hertfordshire, UK) at the University of Liverpool's Centre for Genomic Research. DNA samples from isolates M1 and C1 were each sequenced on a quarter plate; the sample from isolate C2 was sequenced on two quarter-plates. Approximately equal quantities of DNA from isolates M2, M3, M4 and C1-2a were pooled and sequenced on a full Titanium plate.

Analysis of genome sequence and of sequence variants

454 sequence reads were assembled using Newbler 2.0.01.14 (Roche, Welwyn Garden City, Hertfordshire, UK). We combined the sequence data from all MDR-Aci isolates to create a consensus outbreak assembly. The gsMapper component of Newbler was then used to map each set of sequence reads against the consensus assembly. False positive variants resulting from sequencing errors were excluded, generating a set of high-confidence variants. This set was subjected to several additional rounds of filtering using xBASE-NG[17] to generate a set of well-trusted SNPs. During this process, we discarded

1. insertions and deletions
2. variants present in < 90% of mapped reads from the runs with single genomes, or in <25% of reads from the pooled sample.
3. variants with excess coverage, > 1 standard deviation from the mean (i.e. in repetitive regions).
4. variants occurring within 200 bases of a contig boundary.
5. variants occurring in clusters (≥3 SNPs in 1000 base pairs)
6. variants not flanked by good-quality coverage for ≥ 20 basepairs.
7. variants where the ancestral state could not be determined by reference to published genomes using BLASTN

Validation of single nucleotide polymorphisms

All well-trusted SNPs were investigated by polymerase chain reaction (PCR) and Sanger sequencing. The sequences of the primers used for this purpose were as follows: SNP1 TAAGGCAGAACAAAGCGTGA/AATCGGTTCTGAGGTTTGGA (product size 222bp); SNP2

GGTGAACCTTGGTGGTGGTA/AGCTTTAATGGCTGCTCGAA (product size 222bp); SNP3 CATTTCCGAAACCCTCTGAT/AGGCGGTATTTGATGATCTTG (product size 218bp).

PCR products were purified by ethanol precipitation and sequenced on a 3730 DNA analyser (Applied Biosystems, Warrington, UK). The sequences of SNP loci from isolates C1-2b, C1-3a and C1-3b were determined only by sequencing of PCR products.

Results

Description of the outbreak

MDR-Aci was isolated from specimens from five male patients, admitted to the Selly Oak Hospital critical care unit over a six-week period in late 2008. Three patients were military [M1, M2, M3]; two were civilian [C1, C2]. MDR-Aci was also isolated from a wound swab from a military patient [M4] in a nearby trauma ward. Figure 1 shows a time line of the MDR-Aci cases on the critical care unit. The critical care unit is split into a ten-bedded unit and a six bedded-unit, separated by a narrow corridor. Most military patients are admitted to the six-bedded unit, which includes a four-bedded bay (beds 1-4) and a two-bedded bay (beds 5-6), separated by an open thoroughway to the main unit. Patient C1 was first found to be colonized while on the main ten-bedded unit, and was subsequently transferred to the six-bedded unit. The other four MDR-Aci-positive critical-care patients (M1, M2, M3, C2) were cared for exclusively in the six-bedded unit.

All MDR-Aci isolates had an identical profile by PFGE and VNTR analyses and all fell within European clone 1 (Turton, personal communication; data not shown).[18] The antibiotic resistance profile was also identical for all isolates (resistant to meropenem, piptazobactam, amikacin; sensitive to gentamicin and tigecycline).

The first MDR-Aci isolate was from patient M1. The last isolate came from patient C2, who yielded a positive sample in week 7. During our initial epidemiological evaluation, we assumed that all military patients were colonised prior to admission. However, the events leading to colonisation of the civilian patients remained unclear. In particular, several epidemiological scenarios could explain the acquisition of MDR-Aci by patient C2 (Figure 1):

1. Transmission from M1. C2 was nursed in a bed next to M1 during week 2.
2. Transmission from M2. C2 was nursed in a bed next to M2 during week 4.
3. Transmission from M3, who occupied a nearby bed space in the six-bedded unit in the two weeks before MDR-Aci was first isolated from C2.
4. Transmission from C1, who occupied a nearby bed space in the six-bedded unit in the week before MDR-Aci was first isolated from C2
5. Acquisition from an unknown source, such as an environmental reservoir or an unidentified patient or health worker.

Transmission from M4 was rendered unlikely by the lack of proximity to C2 in time and space.

Whole-genome sequencing of outbreak strains

Complete genome sequence data was obtained from the initial MDR-Aci isolates from the six patients plus one additional isolate from patient C1 obtained two weeks after colonisation was first detected (C1-2a) (Table 1). The consensus outbreak assembly output by Newbler comprised 4,110,513 base-pairs, containing 107 contigs ≥500 base-pairs. Average contig size was 38,416 base-pairs, with an assembly N50 of 79,057 base-

pairs. The largest contig was 230,667 base pairs. As predicted from multiplex PCR (reference 8) and PFGE analysis, the outbreak strains align most closely with sequences from other representatives of European clone 1 (data not shown).

Detection and interpretation of SNPs

Several hundred variants were discarded (supplementary data) during the filtering of variants to create high-quality informative SNPs associated with three polymorphic loci (Table 2). The sequences at each SNP locus in each isolate (including those that had not been genome-sequenced) were determined by PCR and Sanger sequencing. For the genome-sequenced isolates, there was complete agreement here with the 454 data. The SNPs were placed in a biological and phylogenetic context by reference to the complete genome sequence of the European clone 1 strain AB0057.[19] This comparison identified the M1 isolate as bearing the ancestral genotype at all three SNP loci.

The first SNP distinguishes all the other outbreak isolates (M2, M3, M4, C1, C2) from the ancestral/M1 state. A second SNP separates the C1 isolate from all the other isolates, while a third SNP differentiates the M3 isolate from all the other isolates. No SNPs were detected between isolates from patients C2, M2 or M4. Interestingly, patients M2 and M4 were injured in the same incident and had similar pathways of care until arrival in Selly Oak Hospital. However, once in Birmingham, patient M4 did not come into close contact with patients M2 and C2.

If we assume that all military patients acquired MDR-Aci before arrival in Birmingham, then SNP 1 must have been acquired before admission and so patient M1 cannot be the source of any of the civilian cases (Figure 1). Transmission of MDR-Aci from C1 or M3 to C2 is ruled out by the very low probability of reversion to the ancestral state for SNPs 2 and 3. Therefore, we conclude that the most parsimonious interpretation of the data is that patient C2 acquired MDR-Aci from patient M2.

Discussion

Current typing systems have provided valuable insights into the epidemiology of MDR-Aci. However, we postulated that whole-genome sequencing might provide more detailed resolution between bacterial isolates from a hospital outbreak. Here, we have shown that closely related MDR-Aci lineages contain SNPs that can shed light on transmission events within a small-scale outbreak and can discriminate between alternative epidemiological hypotheses.

Our analyses support transmission of MDR-Aci from the wound of a military patient M2 to the respiratory tract of a civilian patient C2. However, as MDR-Aci was not isolated from C2 until several weeks after M2 left the adjacent bed, we cannot determine when and how transmission occurred. One possibility is that C2 became colonised when the two patients were nursed together, but that colonization did not reach detectable levels in the sputum until much later. Another possibility is that M2 contaminated the local environment and C2 acquired the organism from the environment only after M2 had left the ward. This latter option would be consistent with a significant role of the environment in transmission of MDR-Aci, as suggested by others.[20-24]

Other uncertainties remain in the epidemiology of this outbreak. In particular, we were not able to determine the source and mode of MDR-Aci transmission to patient C1. The isolate from this patient contained a SNP not present in any of the military isolates and prior to detection of MDR-Aci, this patient never came into close proximity with any

other patients known to be colonized. Curiously, one of the five MDR-Aci isolates from this patient (C1-2a) possessed three additional SNPs not found in any of the other isolates (data not shown). The co-existence of two closely related but distinct lineages of MDR-Aci in samples from the same patient remains puzzling and perhaps reflects two separate acquisition events before arrival in Birmingham.

In conclusion, we have highlighted the potential of whole-genome sequencing in the analysis of hospital outbreaks. However, it is worth stressing that in considering only well-validated SNPs, we have been conservative in our analysis and in future studies, additional phylogenetic analyses (e.g. of short repeats, indels or re-arrangements) twinned with genome finishing methods, such as gap closure, might provide further discrimination between closely related MDR-Aci isolates.

It is also clear that additional studies are needed to benchmark genomic variability within populations of MDR-Aci colonizing individual patients, to determine how frequently SNPs arise within a lineage, to dissect the local, national and global population genomics of *A. baumannii* at the highest resolution possible and to optimise use of this technology in hospital infection control. In addition, this technology is certain to illuminate key biological differences between isolates by revealing the genetic determinants associated with virulence or antibiotic resistance. Furthermore, as improvements in high-throughput sequencing result in reduced costs and increased efficiency, whole-genome sequencing will increasingly come within the reach of clinical microbiology laboratories. It is not hard to imagine a time when genome sequencing replaces gel-based methods as the typing method of choice for bacterial nosocomial pathogens.

Sequence Data

454 sequencing reads have been deposited to the NCBI Short Read Archive under reference SRA010038. Supplementary data is available from http://pathogenomics.bham.ac.uk/acinetobacter/

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Figures and Tables

Figure 1 - Legend

Time line showing bedspaces of individual patients while in the six-bedded bay of the critical care unit. Vertical bars indicate a positive *MDR-Aci* isolate from the patient and their corresponding SNP genotype. Patient C2 had sputum samples sent for microbiological analysis on day 24 and day 42, from which MDR-Aci was not isolated. Patient C1 was initially admitted to the ten bedded main section of the critical care unit, on the same day as patient M2 was admitted to the six bedded section. Patient C1 was first found to be colonized with MDR-Aci two days after patient M2. The arrow shows proposed transmission from M2 to C2.

Table 1

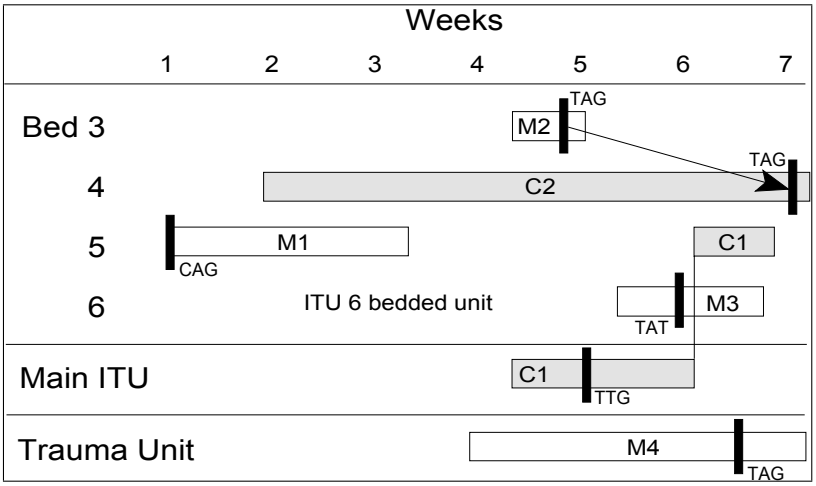
Isolate	*Reads*	*Aligned bases*	*Mean coverage depth*	*SNPs after filtering*
M1	102,493	4115447	9.6	1
C1	68,448	4094862	6.6	1
C2	43,540	3991503	4.3	0
Pool of 4 isolates (M2, M3, M4, C1-2a)	601,802	4117083	53.3 (~13x per isolate)	5

Result of sequencing and mapping alignment showing number of reads generated in each run, the number of nucleotide bases aligned to the reference consensus genome, coverage depth and the number of SNPs detected after filtering.

Table 2

	SNP loci		
	1	2	3
Locus tag	AB57_2551	AB57_2001	AB57_1823
SNP Coordinate	2645863	2093446	1906419
Predicted Product	Two-component heavy metal response regulator	Hypothetical protein	Transcriptional regulator, AraC family
Predicted SNP Effect	Synonymous	Non-synonymous (E to V)	Premature termination at codon 203
	Alleles		
AB0057	C	A	G
M1	C	A	G
M2	**T**	A	G
M3	**T**	A	**T**
M4	**T**	A	G
C1	**T**	**T**	G
C2	**T**	A	G

SNP loci which vary between outbreak isolates are shown. The corresponding annotation for each locus is shown for the ancestral strain AB0057. Alleles in bold demonstrate variation from the ancestral state. The predicted effect of the SNP on each affected protein product is also shown.


Weeks
1
2
3
4
5
6
7
Bed 3
4
5
6
Main ITU
Trauma Unit
M2
TAG
TAG
C2
M1
CAG
C1
ITU 6 bedded unit
M3
TAT
C1
TTG
M4
TAG